Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/IL05/000125

International filing date: 02 February 2005 (02.02.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/577,210
Filing date: 07 June 2004 (07.06.2004)

Date of receipt at the International Bureau: 14 April 2005 (14.04.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

PCT/IL2005/000125
07 APR 2005

PA 1285824

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

February 23, 2005

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *60/577,210*
FILING DATE: *June 07, 2004*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

H. L. Jackson

H. L. JACKSON
Certifying Officer

Attorney Docket No. 26189

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

NATAN et al.

Serial No. NOT YET ASSIGNED

Filed: June 7, 2004

For: **METHOD FOR CRYOPRESERVATION AND STERILIZATION OF BIOLOGICAL MATERIAL**

TRANSMITTAL LETTER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Submitted herewith for filing in the U.S. Patent and Trademark Office is the following **PROVISIONAL APPLICATION:**

(1) Transmittal Letter
(2) Cover sheet for filing **Provisional Application**
(3) 38 page Provisional Application consisting of:
    31 pages Textual Specification,
    3 pages of Claims,
    0 page of the Abstract,
    4 sheets of Drawings;
(4) Check No. 2133 $ 80.00 for filing fee as a small entity;
(5) Postcard for early notification of serial number.

The Commissioner is hereby authorized to charge any deficiency or credit any excess to Deposit Account No. 14-0112.

Respectfully submitted,
NATH & ASSOCIATES PLLC

By: _____
Gary M. Nath
Registration No. 26,965
Lee C. Heiman
Registration No. 41,827
Customer No. 20529

Date: June 7, 2004
NATH & ASSOCIATES PLLC
1030th 15TH Street, NW - 6th Floor
Washington, D.C. 20005
GMN/LCH/ng:PROVAP_trans_LCH

Gary M. Nath (DC, NJ)
Todd L. Juneau (DC, IL)
Ross A. Epstein (CA)*
Marvin C. Berkowitz (DC, GA)
Robert C. Ryan (NV, IL)*
Robert P. Cogan (CA, DC)

Harold L. Novick (DC, MD)++

Irvin A. Lavine (DC), Retired
Donald M. Sandler (MD), Retired*
Patent, Trademark and Copyright Causes
Unfair Competition, Trade Secrets,
Licensing and Litigation

++Of Counsel

NATH & ASSOCIATES PLLC
Attorneys at Law
1030 Fifteenth Street, N.W.
Sixth Floor
Washington, D.C. 20005-1503

TELEPHONE (202) 775-8383
(202) 775-9393

FACSIMILE (202) 775-8396
(202) 822-9409

E-MAIL: IP@NATHLAW.COM
WEB: WWW.NATHLAW.COM

Michelle L. Hartland (VA)*
Lee C. Heiman (CA)*
Jerald L. Meyer (VA)*
Tanya E. Harkins (MD)*
Joshua B. Goldberg (VA)*
Sheldon M. McGee (DC)
Derek Richmond (VA)*
Ryan A. Heck (NV, TX)*
Alvin E. Tanenholtz **
Angela Y. Dai **
Jarrod N. Raphael **

*Practice limited to Matters and Proceedings before Federal Courts and Agencies; not Admitted in DC

** Registered Patent Agent: not Admitted in DC

COVER SHEET FOR FILING U.S. PROVISIONAL APPLICATION UNDER 37 CFR 1.53(c)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Re: New U.S. Provisional Patent Application
For: **METHOD FOR CRYOPRESERVATION AND STERILIZATION OF BIOLOGICAL MATERIAL**
Inventor(s): NATAN et al.
Attorney Docket: 26189

Sir:

Attached hereto is the application identified above, including:

__38__ Pages Application Consisting of:
__31__ Pages of Textual Specification
__3__ Pages of__25__claims
__0__ Page of the Abstract
__4__ Pages of Drawings
__ Executed Inventor's Declaration

The present provisional application names the following inventor(s):1)Yehudit NATAN, Holon, ISRAEL; Tamir KANIAS, Givat Shmuel, ISRAEL.

The Government filing fee* is calculated as follows:
Base Fee (Provisional Application) . . . . . $ 160.00

TOTAL FILING FEE*
(accounting for possible small entity status) . . . $ 80.00

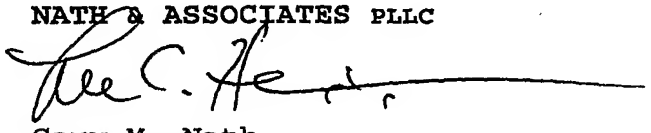
_X_ Reduced by one-half, as applicant(s) is/are a "small entity".

_4_ Sheets of Drawing(s) is/are attached.

_X_ Submitted herewith is a check in the amount of $ 80.00. The Commissioner is hereby authorized to charge any deficiency or credit any excess to Deposit Account No. 14-0112.

Respectfully submitted,

NATH & ASSOCIATES PLLC



Gary M. Nath
Registration No. 26,965
Lee C. Heiman
Registration No. 41,827
Customer No. 20529

Date: June 7, 2004

NATH & ASSOCIATES PLLC
1030 15TH Street NW - 6th Floor
Washington, D.C. 20005
(202)-775-8383
GMN/LCH/ng (ProvisionalAppl.coversheet)

METHOD FOR CRYOPRESERVATION AND STERILIZATION OF BIOLOGICAL MATERIAL

FIELD OF THE INVENTION

This invention relates to the low temperature preservation of biological material, including hypothermic preservation, freezing and lyophilization. More specifically, it relates to the composition of the media in which the biological material is preserved. The invention also relates to a method of preservation of the biological material to improve its survival after storage and/or the cryopreservation process and to a method for the sterilization of lyophilized material.

LIST OF REFERENCES

The following references are considered to be pertinent for the purpose of understanding the background of the present invention:

1. WO 03/020874 – *Improved Method for Freezing Viable Cells;*
2. WO 99/60849 – *Cryopreservation of Human Red Blood Cells;*
3. Fujiki et al. *Mechanistic Findings of Green Tea as Cancer Preventive for Humans.* Proc. Soc. Exp. Biol. Med. 220(4)225-228;
4. US 2004/067157 *Methods for Sterilizing Biological Materials;*

5. WO 2004/009138 *Methods for Sterilizing Milk;*

6. US 5,827,741 *Cryopreservation of Human Adult and Fetal Pancreatic Cells and Human Platelets;*

7. US 6,723,497 *Therapeutic Platelets and Methods;*

8. US 5,869,092 *Prevention of leakage and phase separation during thermotropic phase transition in liposomes and biological cells;*

9. Suganuma et al. 1999 *Green Tea and Chemprevention* Mutation Research 428, 339-344;

BACKGROUND OF THE INVENTION

Biological material is often kept at low temperatures to prevent damage that may be caused from biological processes during storage. For a relatively short period of storage (normally up several to weeks) the material may be kept at low temperatures that are above freezing (hypothermic preservation). For example, red blood cells (RBC) are usually stored for up to 42 days in a refrigerator at about 4°C, after which they must be discarded because RBC recovery falls below acceptable levels.

Preservation at a temperature below 0°C (defined herein as "*cryopreservation*"), allows much longer storage times. Cryopreservation is achievable by freezing or by vitrification. In vitrification, ice-crystals are not formed, however high concentrations of potentially toxic cryoprotectant agents must be added to the biological material. These cryoprotectant agents must be removed before the biological sample is used, in order not to harm the recipient of the biological material. Freezing is also known to cause damage. For example, ice crystals forming in the solution exert extra-cellular mechanical stress. Intracellular stress can be caused for example by osmosis of water into the extra-cellular space, to replace water that is already frozen.

One factor that has a major effect on the success of cryopreservation is the composition of the solution in which the biological material is immersed prior to freezing. Currently many different cryopreservation solutions are known. Normally, such solutions contain a balanced salt solution such as phosphate buffered saline (PBS) and cryoprotectant agents (CPAs). Most freezing solutions comprise intracellular cryoprotectants (DMSO, Glycerol, Ethylene Glycol, etc.) and extra cellular cryoprotectants (Sugars, proteins, carbohydrates such as hydroxy ethyl starch (HES), dextran, etc.). Trehalose, for example, is thought to be protective by binding to lipid polar groups and replacing water. In addition, in WO 99/60849 for example it was claimed that addition of biochemistry altering reagents would reduce hemolysis of RBC during the freeze-thaw cycle.

One of the drawbacks of cryopreservation (both freezing and vitrification) is the need to remove potentially toxic cryopreservation agents before using the cryopreserved biological material. This washing process may damage the cells and (in the case of frozen RBC for example) typically takes about 30 minutes, thus rendering frozen blood supplies an impractical solution for emergency use.

Another drawback of cryopreservation is the need to preserve the frozen biological material at a temperature below -130°C. This is normally done in containers of liquid nitrogen (LN) by either immersion of the biological material in LN or in LN vapor. This adds significantly to the cost of long-term preservation. In addition, incidents are known where the LN in the container evaporated (either due to a malfunction of the container or human error) and the biological materials were damaged.

One method that can overcome this obstacle is lyophilization of the frozen biological material (e.g. WO 03/020874). Lyophilization is a process in which ice crystals are removed by sublimation and desorption, resulting in dry matter. The lyophilized material may be stored at room temperature for a long period of time and be rehydrated for use by simply adding water. Lyophilization results in higher survival rates than air drying or heating, but is still a damaging process.

In order to enhance the biological material's ability to survive the freeze-drying process, intercellular and/or extra-cellular lyoprotectant agents (LPAs) are often added to the biological material. One such LPA is trehalose. It was shown, for example, that loading of platelets with trehalose, or with trehalose and dimethyl sulfoxide, may improve their ability to withstand cryopreservation without premature inactivation (e.g. US 5,827,741 and US 6,723,497).

Epigallocatechin gallate (EGCG) is a polyphenol found naturally in green and black tea. The well-known beneficial effect associated with such tea is attributed, at least in part, to EGCG. Among the mechanisms associated with EGCG's beneficial effects are its ability to function as an antioxidant, its ability to associate with the phospholipids bilayer of the cell membrane (Fujiki et al. 1999) and more. Whilst EGCG is the main constituent of green tea, other polyphenols that are found naturally in green tea, such as epicatechin gallate (ECG) epigallocatechin (EGC) and epicatechin (EC), are also found in green tea and, like EGCG, are considered to be non-toxic. This polyphenols share structural and functional properties with EGCG (Suganuma et al. 1999).

When storing cells or tissue material, there is always the danger of contamination from bacteria, viruses, yeasts, molds, fungi etc., and sometimes the contaminants are present in the biological material when it is first collected. Contaminants are such agents that may damage the biological material during preservation and/or harm the recipient when the product is used (e.g. transfused, injected or eaten).

Among known contaminants are white blood cells (WBC) that are normally present in RBC samples. The presence of WBC is a problem due to graft vs. host disease, in which the grafted WBC attack the recipient's body.

Many methods for sterilization are known in the art including heating and filtration. However, these processes may damage biological material (e.g. when it is sensitive to heat) or prove to be inefficient (e.g. when the biological material is filtered with some contaminants). Other ways for sterilization involve irradiation, mainly gamma rays and UV. For example, gamma radiation is being used for

removal of WBC from fresh blood units. However, gamma radiation can be damaging to radiation-sensitive products. In particular it has been shown that gamma radiation is injurious to red blood cells, platelets and granulocytes (US 2004/067157).

UV radiation is absorbed by liquid, therefore when trying to sterilize material in a liquid a substantial part of the UV radiation is lost, and thus contaminants may remain intact. Thus, in WO 2004/0091938 it was suggested that the reduction of the residual solvent content of the biological material would protect the biological material from ionizing radiation. However, lyophilization of biological material was restricted to non-cellular biological material. Apparently "*sensitive biologicals, such as blood, would lose viability and activity if subjected to freezing for irradiation purposes and then thawing prior to administration to a patient*" (*id.*).

GLOSSARY

The term "*low temperature preservation*" denotes the process of lowering the temperature of biological material to a temperature below about 8°C (or even below 0°C),including hypothermic preservation and cryopreservation. Low temperature preservation may also mean the maintenance of biological material at such temperature. The term "*low temperature preservation solution*" means a solution comprising such components that would allow the biological material to endure the low temperature preservation and if necessary also sustain the biological material. It would normally comprise a balanced salt solution such as phosphate buffered saline (PBS), and other constituents that are known to improve the biological material's ability to withstand low temperature preservation. Hypothermic preservation solutions and cryopreservation solutions are specific examples of low temperature preservation solutions. Accordingly, "*low-temperature-preserved biological material*" means biological material that underwent low temperature preservation. The temperature of such low-

temperature-preserved biological material may also be hypothermic (i.e. below 8°C and above 0°C) subzero (e.g. when it is maintained in a frozen state) or higher than hypothermic temperature (e.g. after thawing or lyophilized biological material that is maintained at temperatures above zero)

The term "*hypothermic preservation*" means low temperature preservation at a temperature above freezing, wherein biological processes are slowed down thus allowing prolonged storage of biological material. The term "*hypothermic preservation solution*" means a solution comprising such components that would allow the biological material to withstand the reduced temperature and the necessary metabolites to sustain its viability. It may include a balanced salt solution such as phosphate buffered saline (PBS) and one or more energy sources (such as sugar, adenine, a phosphate source etc.). Additional components often used in hypothermic preservation solutions are one or more antibiotics and, in the case of blood samples, also an anti-coagulant. An example for such hypothermic preservationsolutions are CPDA-1 (Sodium citrate, Citric acid, Monobasic sodium phosphate and Adenine) , and. ADSOL (Baxter, USA) which are used for hypothermic storage of RBC.

The term "*cryopreservation*" denotes a process of lowering the temperature of biological material from a temperature that is above the freezing temperature of the biological material (or the solution in which it is immersed) to a temperature that is below that freezing temperature. Cryopreservation encompasses freezing, vitrification and lyophilization. Cryopreservation may also mean maintenance of the biological sample in a preserved state that was reached by the cryopreservation process. The term "*cryopreservation solution*" refers to any solution or media in which a biological material is immersed before cryopreservation. Typically, cryopreservation solutions contain a balanced salt solution such as phosphate buffered saline (PBS) and at least one (intracellular and/or extra-cellular) cryoprotectant agent (CPA) or intracellular and/or extra-cellular lyoprotectant agent (LPA). A cryopreservation solution may be a freezing solution, a lyophilization solution and/or a mixture of such solutions. :

The term "*freezing*" denotes a process of cryopreservation that causes the formation of ice crystals within the frozen material. The term "*freezing solution*" refers to any solution or media in which a biological material is immersed before freezing. It comprises constituents that are intended to maintain the biological material whilst reducing the damage caused to the biological material by freezing and/or thawing. Freezing solutions normally comprise intercellular and/or extra-cellular CPAs.

The term "*lyophilization*" or "*freeze-drying*" denotes a process of cryopreservation in which the biological material is being frozen and dried. Thus, in the present invention wherever biological material is said to be freeze dried or lyophilized, this may mean that at least two steps were executed, one of which for freezing the sample and the other for drying. The term "*lyophilization solution*" refers to any solution or media in which a biological material is immersed before lyophilization. Typically, lyophilization solutions contain constituents that are intended to maintain the biological material whilst reducing the damage caused to the biological material by freezing, drying and/or rehydrating. Lyophilization solutions normally comprise one or more intercellular and/or extra-cellular LPAs.

The term "*cryoprotectant agent*" denotes any agent that improves the post thaw viability of a biological material. Intracellular CPAs are thought to replace water inside the cells, thus preventing crystallization therein, to enlarge the un-frozen fraction of the frozen solution, to buffer osmolarity and/or to stabilize the membrane and prevent mechanical damage caused by ice crystals. Examples of CPAs are DMSO, Glycerol, Ethylene Glycol, Poly Ethylene Glycol, propylene glycol, sugars, such as sucrose, dextrose, trehalose, and proteins, carbohydrates such as hydroxy ethyl starch (HES), dextran, etc.

The term "*lyoprotectant agent*" means a substance that stabilizes biological material during lyophilization and/or during storage and may result in higher viability rates. Examples of LPAs include antioxidants, sugars, membrane stabilizers, high molecular weight molecules, etc.

The term "*biological material*" denotes any a material (natural or man made) comprising material of biological source such as samples or tissue taken from an organism, including: cells, portions of cells, groups of cells, tissues, organs, and biological fluids. Such biological material may comprise, without limitation, any of the following: whole blood or fractions thereof, red blood cells (RBC), white blood cells, umbilical cord blood (UCB) or fractions thereof, UCB cells, stem cells, bone marrow, oocytes, sperm, ova, embryos, cartilage, ovary, heart, skin, kidney, liver, lung, eukaryotes and prokaryotes, including bacteria and yeast, etc. Additionally, biological material may also comprise whole multicellular organisms that are capable of surviving cryopreservation such as nematodes. Fractions of blood may comprise any fraction of blood comprising blood cells (white and/or red), plasma and/or solutes and/or sub-cellular components (e.g. fractions of cells, such as platelets, components of degraded cells, etc.), proteins, lipids, antibodies, etc. Biological material may be taken to denote specifically "*Membranous biological material*" namely, biological material having a biological membrane (synthetic or not), including nucleus free cells (e.g. RBC), parts of cells (e.g. platelets), artificial or semi-artificial material such as liposomes. In some cases it may be preferable to use membranous biological material that is essentially free of nucleotide-containing biological material that is not a contaminant. Such biological material would be, for example, an RBC or platelet enriched fraction of blood, samples of liposomes, etc. The membranous biological material is considered essentially free of nucleotide-containing biological material when it is enriched such that at least 50% of the nucleotide-containing cells are removed, preferably at least 70% or even 90% and more are removed. Optimally, the membranous biological material is 100% free of nucleotide-containing biological material.

In this invention, "*liposomes*" mean hollow lipid vesicles. They may be used to entrap the substance to be delivered within the liposomes, or the drug molecule of interest can be incorporated into the lipid vesicle as an intrinsic membrane

component, rather than entrapped into the hollow aqueous interior, or electrostatically attached to the aggregate surface.

The term "*viable biological material*" means biological material comprising some viable cells or fractions of cells that are metabolically active or would become metabolically active if low temperature preservation is ceased. Preferably in viable biological material at least 10% of the cells are viable cells, or at least 30% or 50%. In the case of RBC for example a preferred percentage of viable cells may be in some cases more than 75%. Low temperature preservation may be ceased using many different processes that should be chosen to suit the method of low temperature preservation and the nature of the biological material, including raising the temperature of the biological material, hydration of lyophilized biological material and/or removal of solutes.

The term "*contaminants*" is taken to mean material that is radiation sensitive in a lyophilized state and that may be present in biological material, either at the time of harvesting or that may contaminate the biological material at a later time, and that may damage the biological sample, its recipient or otherwise interfere with its use. Such contaminants may be any type of biological material or artificial material such as engineered nucleic acid sequences, viruses, bacteria, fungi, parasites, yeasts, molds, etc. "*Active contaminants*" are such agents that may damage the biological material and their radiation sensitivity is such that upon irradiation at least a portion of the contaminants becomes inactive (or damaged), for example by becoming less likely to form a colony (e.g. bacteria) or less likely to infect target cells (in case of viruses). "*Reduction of active contaminants*" means that at least a portion of the active contaminants in a given sample become irreversibly inactive, either by degradation or change of conformation, etc.

The term "*polyphenols*" denotes one or more natural and/or synthetic polyphenols that may be naturally found in green tea, including epigallocatechin gallate (EGCG), epicatechin gallate (ECG) epigallocatechin (EGC) epicatechin (EC), in excess of the amount naturally found in the relevant biological material

SUMMARY OF THE INVENTION

The present invention is based, among other things, on the inventors' discovery that EGCG protects biological material during cooling, cycles of freezing and thawing, freeze-drying and hydrating. One of the benefits of using EGCG is that this compound, and to a lesser extent the other polyphenols, are considered beneficial food additives and as such may not need to be removed from the biological material before the biological material is used.

According to one aspect of the invention, a novel low temperature preservation solution is provided, the solution comprising polyphenol, the polyphenol being preferably EGCG.

According to one embodiment, the low temperature preservation solution is a hypothermic preservation solution. Alternatively, the low temperature preservation solution is a cryopreservation solution.

According to another aspect of the present invention, a method is provided for the low temperature preservation of biological material, said method comprising:

(a) providing biological material;

(b) adding a low temperature preservation solution to said biological material, said low temperature preservation solution comprising polyphenol;

(c) cooling the biological material;

such that low-temperature-preserved biological material is obtained.

Preferably, the polyphenol of the above method for the low temperature preservation of biological material is EGCG. It was shown by the inventors that when the biological material comprised platelets or RBC and/or bacteria, the resultant low-temperature-preserved biological material was viable biological material.

The method of the present invention may be applied for hypothermic preservation, freezing and/or lyophilization, depending among other things on the purpose of the biological material, the intended duration of storage, and the composition of the biological material.

It is appreciated that when the biological material is lyophilized and intended to be stored at a given temperature (e.g. room temperature), it is preferred that the low temperature preservation solution will not contain agents (e.g. CPAs or LPAs) that are liquid at the given temperature.

When the low temperature preservation is lyophilization, the method of the present invention may further comprise:

(d) irradiating the biological material, such that the amount of active contaminants in the membranous freeze dried biological material is reduced, and the biological material remains viable biological material.

According to another aspect of the present invention, biological material is provided, said biological material being obtainable by carrying out the method of the present invention.

According to another aspect of the present invention, low-temperature-preserved biological material is provided, said biological material comprising viable biological material and added EGCG.

According to yet another aspect, the present invention provides a method for the reduction of active contaminants in membranous freeze dried biological material, said method comprising:

(a) providing membranous freeze dried biological material;

(b) irradiating said membranous freeze-dried biological material;

such that the amount of active contaminants in the cellular freeze dried biological material is reduced, and the biological material remains viable biological material.

Preferably, the membranous freeze dried biological material being irradiated in the above method is essentially free of nucleotide containing biological material. Accordingly also lyophilized membranous biological material is provided, being essentially free of active contaminants.

DETAILED DESCRIPTION OF THE INVENTION

According to the first aspect of the present invention a low temperature preservation solution is provided, comprising polyphenol. Preferably, the polyphenol is one or more of epigallocatechin gallate (EGCG), epicatechin gallate (ECG) epigallocatechin (EGC) and epicatechin (EC). Most preferably, the polyphenol is EGCG.

A person skilled in the art of the invention would be able to adjust the contents of the low temperature preservation solution to the duration and purpose of low temperature preservation and the type of biological material to be preserved. For example, when the purpose is hypothermic preservation, the low temperature preservation solution is a hypothermic preservation solution. When the low temperature preservation is cryopreservation, the low temperature preservation solution is a cryopreservation solution. In such case the solution may be for example a freezing solution (in which case the biological material is frozen) or a lyophilization solution (in which case the biological material is lyophilized).

Preferably, the low temperature preservation solutions would not contain substances that would need to be removed from the biological material prior to its use. Thus it is preferable that the low temperature preservation solutions would not contain intracellular and/or extra-cellular CPAs or intracellular and/or extracellular LPAs.

According to another aspect of the present invention a method is provided for the low temperature preservation of biological material, said method comprising:

(a) providing biological material;

(b) adding a low temperature preservation solution to said biological material, said low temperature preservation solution comprising polyphenol;

(c) cooling the biological material;

such that low-temperature-preserved biological material is obtained.

A person skilled in the art of the invention would appreciate that the adding of the low temperature preservation solution may be done in one or more steps such that one or more of the solutions' components would be added separately. Preferably, the low temperature preservation solutions would not contain substances that would need to be removed from the biological material prior to use. Thus it is preferable that the low temperature preservation solutions would not contain intracellular and/or extra-cellular CPAs and/or intracellular and/or extra-cellular LPAs. The polyphenol of the above method may be one or more of epigallocatechin gallate (EGCG), epicatechin gallate (ECG) epigallocatechin (EGC) and epicatechin (EC). Most preferably, the polyphenol is EGCG.

The method of the invention may be applied for cryopreservation, in which case the low temperature preservation solution is a cryopreservation solution and the cooling of step (c) is by cryopreservation. More specifically, the method of cryopreservation may be freezing in which case the cryopreservation solution is a freezing solution or the method may be lyophilization in which case the cryopreservation solution is a lyophilization solution.

In accordance with the present invention it is also possible to sterilize the biological material such that active contaminants are reduced or eliminated. This is based on a surprising finding of the inventors that, although it was commonly accepted that red blood cells would not survive both lyophilization and irradiation, the inventors did manage to reduce the amount of contaminants in lyophilized RBC or platelets and still have viable RBC or platelets. Thus the method may further comprise:

(d) irradiating the biological material, such that the amount of active contaminants in the membranous freeze dried biological material is reduced, and the biological material remains viable biological material;

In some cases, the biological material may be free of active contaminants before irradiation, in which case the present invention would ensure the lack of contaminants and thus negate the need to check for active contaminants. It is appreciated that the biological material may comprise nucleotide (or nucleus) containing cells. In such case, the method of the invention may also reduce these cells as unwanted contaminant. This may be useful in blood samples in order to remove the white blood cells (WBC) whilst retaining viable RBC and/or platelets, which would reduce the adverse immunological reaction of the host to the WBC in the blood. In other cases, it would be preferable that the frozen biological material be such that is essentially free of nucleotide containing biological material.

According to yet another aspect of this invention low-temperature-preserved biological material is provided, comprising viable biological material and polyphenol. The polyphenol may be one or more of epigallocatechin gallate (EGCG), epicatechin gallate (ECG) epigallocatechin (EGC) and epicatechin (EC). Preferably, the polyphenol is EGCG.

This low-temperature-preserved biological material may be cryopreserved (including frozen and/or lyophilized). In one preferred embodiment, the lyophilized biological material comprises RBC. In another preferred embodiment, the lyophilized biological material comprises platelets. In yet another preferred embodiment, the lyophilized biological material comprises bacteria. In yet a further preferred embodiment, the lyophilized biological material is essentially free of active contaminants. In fact, the biological material of the present invention may be such that is obtainable by the performance of any of the methods described above.

The biological material of this invention may accordingly be provided in a hypothermic, frozen or freeze-dried state. It may also be provided in a revived

viable form, after having been warmed, thawed and/or hydrated using any method known in the art that is compatible with the type and condition of the biological material.

According to yet an additional aspect, a method is provided for the reduction of active contaminants in membranous dried biological material, comprising:

(a) providing membranous dried biological material;

(b) irradiating said membranous dried biological material;

such that the amount of active contaminants in the membranous dried biological material is reduced, and the biological material remains viable biological material.

Preferably, the amount of active contaminants in the membranous freeze dried biological material is reduced to none. In a preferred embodiment of this method, the membranous dried biological material comprises RBC. In another preferred embodiment of this method, the membranous dried biological material comprises platelets. It is also preferred that the dried biological material would be freeze dried biological material, however, any viable dry biological material may be subject of the method, provided that it would comprise viable biological material. It is appreciated that the biological material in this method may comprise nucleotide (or nucleus) containing cells. In such case, the method of the invention may also reduce these cells as unwanted contaminants. This may be useful in blood samples in order to remove the white blood cells (WBC) whilst retaining viable RBC and/or platelets, which would reduce the adverse immunological reaction of WBC in the blood to the host. In other cases, it would be preferable that the frozen biological material be such that is essentially free of nucleotide containing biological material.

Irradiation of biological material in any of the methods of this invention may be done using any form of ionizing radiation, including, gamma, UV and visible light. One benefit of the present invention over current gamma ray irradiation of blood is that whilst current irradiation is done in a liquid state, the present invention

teaches irradiation in a solid state. This may allow reduction of use of or exposure to the ionizing radiation (including gamma rays).

Thus, the present invention provides, according to one further embodiment also dry (preferably lyophilized) membranous biological material being essentially free of contaminants. Examples for such dry membranous biological material are samples comprising RBC and/or platelets. The dry membranous biological material may also comprise a polyphenol, being preferably EGCG.

Freeze-drying and cryopreservation in accordance with the present invention may be carried out in any method suitable to the biological material in question.. The freezing of above methods of the present invention can be done in any method or apparatus known in the art. A preferred example would be using a directional freezing device such as that which is described in WO 03/020874. Nevertheless, any freezing method which allows cryopreservation of biological material may be used, including using mechanical freezers, stepwise freezing apparatus, the Planner freezing apparatus etc. Likewise, the biological material may be treated additionally in one or more methods known in the art, such as those described in US 5,827,741, US 6,723,497 or US 6,723,497.

In the following some non-limiting examples are provided, showing how the present invention may be practiced..

BRIEF DESCRIPTION OF THE DRAWINGS

In order to understand the invention and to see how it may be carried out in practice, preferred embodiments will now be described, by way of non-limiting example only, with reference to the accompanying drawings, in which:

Fig. 1. is a chart illustrating the survival of erythrocytes after freezing and thawing (freeze thawing) at different cooling rates with two different solutions at a volume of 9 ml.

Fig. 2. depicts photographs, taken using a light microscope, showing of differential staining of RBC (erythrocytes) with May-Gruenwald Giemsa. **A1 to A4** show RBC mixed 1:1 with a freezing solution containing 30% (w/v) Dextran and 51.5µM EGCG. **A1** shows fresh erythrocytes(X1000), **A2**(X1000) and **A3**(X400) are of frozen thawed erythrocytes and picture **A4**(X1000) is of freeze-dried erythrocytes, after rehydration. **B1to B3**(X1000) depict erythrocytes frozen only with saline. **B1** shows fresh erythrocytes, **B2** and **B3** show frozen thawed erythrocytes.

Fig. 3. shows light microscopy pictures taken after freeze thawing of 9ml of erythrocytes at a cooling rate of 1000°C/min. **A and B** were frozen with a solution composed of Dextran and EGCG. **C and D** were frozen with saline.

Fig. 4. is a chart illustrating the survival rate of erythrocytes after freeze thawing at a volume of 50ml with a freezing solution containing Dextran and EGCG at different cooling rates.

Fig. 5. is a chart illustrating the percentage of erythrocytes and hematocrit after freezing 200ml at a cooling rate of 315°C/min in a conventional freezing bag.

Fig. 6. is a chart illustrating the mononuclear cells (MNC) derived from umbilical cord blood (UCB) frozen with different solutions and then thawed or dried.

Fig. 7. is a chart illustrating MNC derived from UCB after freeze thawing and freeze drying with different concentrations of EGCG.. X1 EGCG = 51.5µM EGCG; HSA & Trehalose were in a concentration of 0.1M Trehalose and 12.5%(w/v) HSA in PBS (Ca^{+2} & Mg^{+2} Free).

EXPERIMENTS

Materials and Methods

Unless otherwise noted, all materials were purchased from Sigma Inc. (St. Louis. Missouri, USA).

I. EGCG Experiments

1. Red Blood Cells (RBC)

Preparation of Packed Red Cells

Red blood cells (RBC) were separated from whole blood received from the Israeli Blood Bank. The blood was centrifuged for 10 minutes at 1500g and the plasma was discarded. The pellet was suspended in an equal volume of phosphate buffered saline (PBS), and centrifuged again at 1500g for 10 minutes. Afterwards the supernatant was discarded, and an equal volume of cryopreservation solution selected from the solutions in Table I was added to the packed RBC.. Different volumes (2.5-50ml) of the RBC suspension were transferred to a 16-25mm diameter glass test tube (Manara, Israel). Experiment of freezing 200ml of RBC suspension was done in a 2000ml freezing bag (Baxter-Fenwal, USA). Samples were then either frozen and thawed or lyophilized and rehydrated as described below.

Table I **Components of Cryopreservation Solutions**

Components: Solution name	PBS*	Autologous Plasma	Dextran**	HSA***	Trehalose	EGCG (mM)
Plasma	-	100%	-	-	-	-
Saline	100%	-	-	-	-	-
Dextran	100%	-	30%(w/v)	-	-	-
HSA + Trehalose	50%	-	-	50%	0.1M	-
Trehalose	100%	-	-	-	0.1M	-
EGCG	100%	-	-	-	-	51.5
EGCG + Dextran	100%	-	30%(w/v)	-	-	51.5
EGCG + HSA + Trehalose	50%	-	-	50%	0.1M	51.5
EGCG + Trehalose	100%	-	-	-	0.1M	51.5

* phosphate buffered saline)(Ca^{+2} and Mg^{+2} free)

** 40,000 Da.

*** Human Serum Albumin

Freezing

Glass test tubes containing RBC were frozen in a modified MTG freezing apparatus, as described in WO 03/020874 (manufactured by IMT, Israel). The thermal gradient in the cooling unit was set to 5.5°C/mm or 10.5°C/mm or 15.5°C/mm (as detailed below) with final temperatures of -50°C, -100°C or -150°C. The rate of test tube insertion (interface velocity) was 0.5, 1, 1.5 and 3mm/sec, yielding cooling rates of 165, 330, 500, 1000 or 2000°C/min. During freezing the test tubes were rotated at a rate of 56 rpm. Subsequent to freezing, biological material was taken from each test tube to test for cell viability.

Lyophilization and Rehydration

Lyophilization was performed in a commercial lyophilizer (Labconco, USA) with a chamber temperature of -35°C and a condenser temperature of -82°C for 72 hours. The biological material was rehydrated by adding ultra pure water (ddH2O) to reach the sample's original volume.

Thawing

Frozen (but not lyophilized) material was thawed by immersion in a water bath at 37°C for until it melted. Melting was observed visually.

Assessment of Cell Survival

a. Cell count

Cells were counted using the PENTRA 60 cell counter (ABX, France), in accordance with the manufacturer's manual. This device provides a complete cell count (CBC) that includes parameters such as: cell number, hematocrit, mean cell volume, hemoglobin, etc.

b. Morphology Evaluations

Morphology evaluations of the erythrocytes were done by observation of a 5µl drop of red blood cells under a light microscope (Zeiss, Germany) and by differential staining of May-Gruenwald Giemsa stains. Cell suspensions were spread on a glass slide, and the slides were left to air dry. Then slides were fixed in absolute methanol for 2-3 minutes at room temperature. Afterwards, slides were stained for 15 minutes in May-Gruenwald stain (freshly diluted with an equal volume of distilled water, pH6.8). After 15 minutes the slides were stained for 10 minutes in Giemsa stain (freshly diluted with distilled water (1:9 ratio), pH6.8). Then the slides were washed in running tap water and left for 3-4 minutes in distilled water. The slides were then allowed to dry and were observed under a light microscope (Zeiss, Germany) using immersion oil.

Example 1 **Cooling rate 1,000°C/min**

Table II shows survival of RBC after freezing cells at 1,000 °C/min. Column A depicts the results for cells that were frozen and thawed but not lyophilized. Columns B depicts the results for cells that were frozen and lyophilized, and were then rehydrated using ultra pure water. The results are presented as the percentage of cells that survived from the total fresh cells counted. In these experiments the cell suspension volume was 2.5ml. **Figure 2** shows

picture of samples that were stained using May-Gruenwald Giemsa as described above.

Table II **RBC survival after Cryopreservation at 1,000 °C/min**

Solution	A Thawed	B Rehydrated
PBS	53.08	49.17
Dextran	98.7	69.95
HSA + Trehalose	97.7	55.20
Trehalose	89.04	-
EGCG	84.53	49.19
EGCG + Dextran	100	70.80
EGCG +HSA+Trehalose	98.6	-
EGCG + Trehalose	-	52.04

Example 2 **Cooling rate 2,000°C/min**

Table III shows survival of RBC after freezing cells at 2,000°C/min. Column A depicts the results for cells that were frozen and thawed. Column B depicts the results for cells that were frozen, lyophilized, and then rehydrated using ultra pure water. The results are presented as the percentage of cells that survived from the total fresh cells counted. In these experiments the cell suspension volume was 2.5ml

Table III **RBC survival after Cryopreservation at 2,000°C/min**

Solution	A Thawed	B Rehydrated
PBS	72.92	71.02
Dextran	98.28	71.92
HSA + Trehalose	99.26	70.79
Trehalose	93.24	-
EGCG	72.92	-
EGCG + Dextran	100.00	72.64
EGCG +HSA+ Trehalose	96.20	-
EGCG + Trehalose	93.24	83.94

Under a light microscope or after May- Gruenwald Giemsa stains (**Figure** 2), RBC that were frozen with EGCG and dextran appeared morphologically normal whereas in all other groups many irregular shaped cells were observed, as

well as a lot of ghosts (i.e. cells whose membrane was damaged and the hemoglobin has leaked out but that did not undergo lysis). Apparently Pentra60 counts ghosts, therefore the additional morphological evaluations are important.

Example 3

In this experiment RBC were frozen at a volume of 9ml using the MTG freezing apparatus at different cooling rates of 500, 1000, 2000 and 2500°C/min. Freezing solution was composed of 30% (w/v) Dextran and 51.5μM EGCG in PBS (calcium and magnesium free). Samples were thawed in a water bath at 37°C. After thawing, cells were counted using the Pentra60 (ABX, France) and morphological observation was conducted using May-Gruenwald Giemsa stain and light microscopy.

Results are shown in **Figures 1 and 3**, wherein we can see that samples frozen with a solution composed of EGCG and dextran had higher viability rates as detected by the Pentra60 machine. In addition, morphological evaluations also showed that RBC frozen with EGCG and dextran had a normal morphological appearance.

Example 4

In this experiment RBC were frozen at a volume of 50ml. Freezing was done using the MTG freezing apparatus at different cooling rate of: 165, 330, 500 and 1000°C/min. Freezing solution was composed of 30% (w/v) dextran and 51.5μM EGCG in PBS (calcium and magnesium free). Thawing was done in a water bath at 37°C. Results are shown in **Figure 4**. The highest survival rate was achieved at a cooling rate of 330°C/min. This cooling rate is lower than that of the best cooling rate in previous experiments. This difference may be due to the significantly high volume of the sample. In large volume the heat transfer is slower; therefore at very high cooling rates a more significant thermal gradient develops across the sample. One method of avoiding these temperature differences and achieving better heat transfer along the sample is the use of lower cooling rates.

Example 5

In this experiment we have frozen RBC in a cell suspension of 200ml. Freezing solution was composed of 30% (w/v) dextran and 51.5μM EGCG in PBS (calcium and magnesium free). The cell suspension was put in a 2000ml volume freezing bag (Baxter-Fenwal, USA). The bag was placed in an MTG freezing apparatus. Freezing was done at a cooling rate of 330°C/min. Thawing was done as before in a water bath at 37°C. Cells were counted using the Pentra60 (ABX, France). Results are shown in **Figure 5** as a percentage of the fresh cell number and hematocrit. We can see that almost 80% of the cells survived when freezing 200ml of RBC suspension. This high a viability rate in such a large volume freezing without any intracellular cryoprotectants has been regarded as impossible, until now.

2. Mononuclear Cells (MNC) Derived from Umbilical Cord Blood (UCB)

Blood Separation

Blood was separated on ficoll-paque gradient for 30 minutes at 1000g. Afterwards the mononuclear layer was drawn out and washed twice in Phosphate Buffered Saline (PBS) (Calcium and Magnesium free) for 10 minutes at 200g. one of the following freezing solutions were added to the pellet:

1) 0.1M Trehalose, 12.5% (w/v) HSA in PBS (calcium and magnesium free).
2) 0.1M Trehalose, 12.5% (w/v) HSA and 51.5μM epigallocatechin gallate (EGCG) in PBS (calcium and magnesium free).
3) 12.5%(w/v) HSA and 51.5μM EGCG in PBS (calcium and magnesium free).
4) 51.5μM EGCG with 0.1M trehalose PBS (calcium and magnesium free).
5) 30% (w/v) Dextran with 51.5μM EGCG in PBS (calcium and magnesium free).

Freeze thawing and freeze drying

2.5ml of cell suspension were put in a 16mm diameter glass test tube (Manara, Israel) and frozen using the MTG freezing apparatus (IMT, Israel). The

temperature gradients were 1.5°C/mm and 0.428°C/mm, velocity was 0.2mm/sec resulting in a cooling rate of 18°C/min and 5.1°C/min respectively. The first cooling rate was applied until the seeding temperature was reached. The samples were rotated at 56 rounds per minute (RPM).

After freezing samples were plunged into liquid nitrogen (LN), and then samples were either thawed at 37°C in a water bath or put in a commercial lyophilizer (Labconco, USA) with a shelf temperature of -35°C and a collector temperature of -80°C. After drying the cells were rehydrated with ultra pure water at 37°C. Cells that were frozen and did not undergo lyophilization were thawed in a water bath at 37°C.

Assessment of Cell Survival

Cells were counted using the PENTRA 60 cell counter, as described above. Viability assessments were done by evaluating the membrane integrity of the cells using SYBR14 plus Propidium Iodide (PI) (Molecular Probes, USA) live/dead fluorescent staining. Both stains are nucleic acid stains, SYBR14 is a membrane permeable molecule and PI can enter the cell only if the membrane is damaged.

Results

Example 6

The viability of the cells after freezing and freeze drying with different freezing solutions (as described above) was assayed. **Figure 6** shows the viability rates as demonstrate by membrane integrity of the cells. **Figure 6** shows that the solution composed of EGCG and trehalose gave best results after freeze thawing and freeze drying.

Example 7

The effect of different EGCG concentrations in a solution comprising EGCG and trehalose was evaluated. The EGCG concentrations were: 51.5 (X1

EGCG), 128.75 (X2.5 EGCG), 257.5 (X5 EGCG) and 515μM EGCG (X10 EGCG), each with 0.1M trehalose in PBS (Ca^{+2} and Mg^{+2} free). As a control we used a solution composed of 0.1M trehalose and 12.5(w/v) HSA. **Figure** 7 indicates that viability increased with EGCG concentration after freeze thawing and after freeze drying.

II. Irradiation Experiments

Example 8

The effect of irradiation on Lyophilized red blood cells (RBC) was evaluated in this experiment.

The freezing solution was composed of 30% (w/v) dextran in PBS (Ca^{2+} and Mg^{2+} free). Packed RBC were mixed at a ratio of 1:1 (v/v) with the freezing solution. Freezing was done at a cooling rate of 1000°C/min; (thermal gradient) G= 5.5°C/mm, V=3mm/sec. The samples were also rotated at 56 RPM (rounds per minute). After freezing, samples were put in a lyophilizer (Labconco, USA) for 3 days (shelf temp. –35°C, condenser –80°C). After 3 days of lyophilization one sample was placed in a petri dish and exposed to UV radiation for 1 hour and the other was protected from light. After 1 hour the samples were rehydrated with ultra pure water to their original volume and were counted using the Pentra 60 (ABX, France).

Table IV The effect of UV exposure on the survival of lyophilized RBC

	Lyophilized RBC	
	exposed to UV	not exposed to UV
Amount of cells	52.04%	57.6%
hematocrit	29.2%	35.5%

The results are shown as a percentage of the fresh sample, before freezing.

As seen in Table IV the sample that was exposed to UV exhibited a slightly lower survival rate than that of the sample that was not exposed to radiation. Since

EGCG was shown above to improve the cells' survival in the freeze-drying – thawing treatments, in the following experiments EGCG was added to the biological samples.

Example 9

In this experiment packed RBC were frozen with a freezing solution containing: 30% (w/v) dextran and 128.7μM EGCG (Cayman Chemical, USA). The freezing solution and the packed RBC were mixed in a ratio of 1:1 (v/v). 2.5ml of the cell suspension were put in 16mm diameter glass test tubes (Manara, Israel). A total of 4 test tubes were frozen. The samples were frozen at a cooling rate of 1000°C/min; (thermal gradient) G= 5.5°C/mm, V=3mm/sec. The samples were also rotated at 56 RPM.

After freezing, samples were placed in liquid nitrogen. After passage of varying time periods (between 1/2 hour to a few weeks) samples were placed in a lyophilizer (Labconco, USA) with a shelf temp was –35 °C, condenser temp was –86 °C) for 72 hours. Then samples were transferred to a 60mm petri dish, 2 samples were exposed to UV for 1 hour and during that 1 hour the other 2 samples were covered with aluminum foil to prevent exposure to light. All samples were then rehydrated with 2ml of pure water at 37 °C and compared using the PENTRA 60 counter (ABX, France). Results are presented as compared to the parameters of fresh RBC in a freezing solution including EGCG.

Table V The effect of UV radiation on lyophilized RBC survival

	Lyophilized RBC	
	no UV treatment	UV treatment
Cells number	58.11%	54.05%
Hematocrit	43.02%	45.95%

Results are shown as percentage of the fresh values of the same samples

As can be seen from Table V, freeze-dried cells were less viable and had a lower hematocrit than fresh cells. However, these parameters were only slightly affected by UV irradiation.

Example 10

E. coli were placed in LB medium: 10gr Bacto-tryptone (Difco, USA), 5 gr yeast extract (Difco, USA), 10 gr NaCl, in 1 liter distilled water. The total volume of 10ml was divided to two batches of 5ml each. To the first batch of E. coli in LB medium we added 5ml of freezing solution composed of 30%(w/v) dextran and 128.7μM EGCG (Cayman Chemical, USA) in PBS (Ca^{+2} and Mg^{+2} free). The other batch was left un-touched. Cell-suspension samples of 2.5ml each (two from each batch) were put in 16mm diameter glass test tubes (Manara, Israel), such that a total of 4 test tubes were prepared. The test tubes were frozen at 1000°C/min (from 5 to -50°C at a velocity of 3mm/sec and with 56 RPM. After freezing was completed the test tubes were placed in liquid nitrogen. Afterwards, the 4 test tubes were placed in a lyophilizer (Labcono, USA) for 72 hours. After lyophilization was completed the "powdered" cells from each test tube were scraped into a petri dish. Two petri dishes (one representing each batch) were exposed to UV radiation for 1 hour (the petri dishes were placed opened under a UV lamp) and the other two petri dishes were left unexposed to radiation (covered with aluminum foil for protection from light). After 1 hour 2ml of double distilled water at 37°C were added to each dish. From each dish 3 petri dishes with agar were plated. The following Agar plates protocol was used: 10gr Bacto-tryptone, 5gr yeast extract, 10gr Na^{+} Cl^{-}, 10gr agar (BD, USA). Water was added to a volume of 1 liter, autoclaved, cooled to 65°C and poured into petri dishes. A total of 12 petri dishes were incubated at 37°C for 24 hours. The next day colonies were counted. Table VI depicts the number of colonies grown on the agar petri dishes.

Table VI Number of E. coli colonies after being frozen with different freezing solutions and lyophilized

E. coli frozen in LB		E. coli frozen with dextran and EGCG	
------	UV	------	UV
36	0	152	0
24	0	220	0
16	0	>200	0

As seen in Table V, E coli colonies were observed only in the plates of the un-radiated bacteria. No colonies were observed in the agar plated with lyophilized cells that were radiated. In addition, the addition of Dextran and EGCG improved the bacteria's survival of lyophilization.

Example 11

10ml of E. coli in LB medium was centrifuged at 800g for 10 minutes. To the resultant pellet 10ml of freezing solution composed of 30% (w/v) dextran and 128.7μM EGCG (Cayman Chemical, USA) in PBS (Ca^{+2} and Mg^{+2} free) were added. This solution was then mixed in a volumetric ratio of 1:1 with packed RBC. 2ml of RBC-coli were put in a petri dish; a total of 4 like dishes were prepared. 2 petri dishes were exposed to UV for 1 hour and the other 2 were not. After 1 hour cells from each group were plated on three agar plates that were placed in a 37°C oven for 24 hours.

From the remaining RBC-coli mixture four test tubes were prepared, each containing 2.5ml. The test tubes were frozen at 1000°C/min (from 5 to -50°C at a velocity of 3mm/sec and with 56 RPM and then placed in a lyophilizer for 72h. After lyophilization one test tube from each group was exposed to UV radiation for 1 hour. After 1 hour 2ml of ddH_2O was added and from each group 3 agar plates

were seeded and placed for 24 hour in 37°C oven for 24 hours. The results are depicted in Table VI.

Table VII The effect of UV radiation on the survival of E.coli in lyophilized or fresh samples comprising RBC

Fresh RBC and E. coli		Lyophilized RBC and E. coli	
------	UV	------	UV
>200	>200	>200	0
>200	>200	>200	0
>200	>200	>200	0

As seen in Table VII irradiation in the liquid state had no measured effect on E. coli , as in all plates more then 200 colonies were observed. However, when radiated in a dry (lyophilized) state no colonies were observed after 24 hours in incubation.

Example 12

A unit of fresh platelets was received from the Israeli blood bank. Platelets were added to an E.coli pellet (E.coli in LB medium that was centrifuged for 10 minutes at 2000g). The platelets-E.coli solution was mixed at a ratio of 1:1 (v/v) with a freezing solution composed of 30% (w/v) Dextran (40000 Dalton; Amersham Biosciences, USA) and 515μM EGCG (Cayman, USA) in PBS (calcium and magnesium free). Two samples, 2.5ml each, of platelet suspension were put in a 60mm petri dish. One dish was exposed to UV radiation for 1 hour, and the other was left untouched, covered in aluminum foil. After one hour, samples from each petri dish were seeded in agarose and put in an incubator at 37°C for 24 hours. After 24 hours colonies were counted.

Table VIII The effect of UV radiation on the number of E. coli colonies

Batch	exposed to UV	not exposed to radiation
Plateletes & E.coli & freezing solution	269	201

Example 13

Platelets-E. coli solutions were prepared as described in Example 12. The platelets-E. coli solution was divided to two batches, and each batch was mixed at a ratio of 1:1 (v/v) with one of the following freezing solutions: (1) 30% (w/v) Dextran and 515μM EGCG in PBS (calcium and magnesium free); or (2) 30% (w/v) Dextran in PBS (calcium and magnesium free). 2.5ml aliquots of platelet suspension were put in 16mm diameter glass test tubes (Manara, Israel). A total of 4 test tubes were prepared, 2 tubes from each batch. The tubes were frozen in the MTG device at a thermal gradient of 5.5°C/mm and at a cooling rate of 1000°C/min (final temperature was -50°C, velocity was 3mm/sec).

After freezing, all tubes were maintained in liquid nitrogen and later lyophilized for 3 days. The resultant dry cells were scraped into a 60mm petri dish, such that two dishes were prepared from each of the above batches. One dish from each batch was exposed to UV radiation for 1 hour. The other 2 dishes (one from each batch) were untouched, covered in aluminum foil. The contents of each petri dish were rehydrated with 2ml of ultra pure water at 37°C and a sample from each dish was seeded in agarose and incubated at 37°C for 24 hours. After 24 hours colonies were counted.

Table IX **The effect of UV radiation on the number of E. coli colonies grown after being lyophilized**

batch	exposed to UV	not exposed to UV
Platelets - E.coli & Dextran 30%	1	17
Platelets - E.coli & Dextran + EGCG	1	11

As seen in Table IX, UV radiation reduced the number of colonies by more than tenfold.

In order to assess the platelets' survival of the UV irradiation in a dry state, samples of platelets (prepared with EGCG and Dextran as described above) taken after lyophilization and rehydration were compared with those taken after UV irradiation. The platelets were counted using the Pentra 60 (ABX, France) cell counter, and we have observed that 80.38% of the platelets that survived lyophilization also survived UV treatment. This shows the feasibility of UV sterilization of platelets in a dry state.

It is well established that drying biological material may be done in methods other than lyophilization. Such drying may be for example air drying of liposomes (Hincha et al. 2003; Biochemica et Biophysica ACTA. 1612(2):172-177), embryonic kidney cell line and human foreskin fibroblasts cells (Gau et al. 2000; Nature Biotechnology. 18:168-171) etc. It is noted that bacteria may survive the air-drying process (Desmond et al. J Appl Microbiol. 2002;93(6):1003-11) and so can other contaminants. Accordingly, the dry biological material that is irradiated in accordance with the present invention may be dry biological material that was dried in any manner, including lyophilization, heating, and/or air drying and in addition one may use spray drying or nebulizing. It is understood that the method of drying must be selected in such manner that the dry biological material would be viable biological material.

In light of the above experiments, it is realized that radiation of biological samples (such as RBC, platelets, liposomes, etc.) in the dry (lyophilized or otherwise) state is more harmful to the contaminants then to the biological sample of choice.

CLAIMS:

1. A low temperature preservation solution comprising polyphenol.

2. The low temperature preservation solution of Claim 1, wherein the polyphenol is epigallocatechin gallate (EGCG).

3. The low temperature preservation solution of anyone of Claims 1 and 2, being a hypothermic preservation solution.

4. The low temperature preservation solution of anyone of Claims 1 and 2, being a cryopreservation solution;

5. The cryopreservation solution of Claim 4, being a freezing solution.

6. The cryopreservation solution of Claim 4, being a lyophilization solution.

7. A method for the low temperature preservation of biological material, comprising:

(a) providing biological material;

(b) adding a low temperature preservation solution to said biological material, said low temperature preservation solution comprising polyphenol;

(c) cooling the biological material;

such that low-temperature-preserved biological material is obtained.

8. The method of Claim 7, wherein the polyphenol is EGCG.

9. The method of anyone of Claims 7-8, wherein the low temperature preservation is cryopreservation, the low temperature preservation solution is a cryopreservation solution and the cooling of step (c) is by cryopreservation;

10. The method of anyone of Claims 7-8, wherein the cryopreservation is freezing and the cryopreservation solution is a freezing solution and the cryopreservation of step (c) is by freezing.

11. The method of anyone of Claims 7-8, wherein the cryopreservation is lyophilization, the cryopreservation solution is a lyophilization solution the cryopreservation of step (c) is by lyophilization.

12. The method of Claim 11, comprising:

(d) irradiating the biological material, such that the amount of active contaminants in the membranous freeze dried biological material is reduced, and the biological material remains viable biological material;

13. Biological material obtainable by the method of any one of Claims 7 - 12.

14. Low-temperature-preserved biological material comprising viable biological material and polyphenol.

15. The low-temperature-preserved biological material of Claim 14, wherein the biological material is cryopreserved.

16. The cryopreserved biological material of Claim 15, wherein the biological material is frozen.

17. The cryopreserved biological material of Claim 15, wherein the biological material is lyophilized.

18. The low-temperature-preserved biological material of anyone of Claims 14 to 17, wherein the biological material comprises RBC.

19. The low-temperature-preserved biological material of anyone of Claims 14 to 18, wherein the biological material comprises platelets.

20. The low-temperature-preserved biological material of Claim anyone of Claims 14 to 17, wherein the biological material comprises bacteria.

21. The lyophilized biological material of Claim 17, wherein the biological material is essentially free of active contaminants.

22. The lyophilized biological material of Claim 21, comprising one or more of RBC and platelets.

23. The low-temperature-preserved biological material of anyone of Claims 14 - 22, wherein the polyphenol is EGCG.

24. A method for the reduction of active contaminants in membranous dried biological material, comprising:

(a) providing membranous dried biological material;

(b) irradiating said membranous dried biological material;

such that the amount of active contaminants in the membranous freeze dried biological material is reduced, and the biological material remains viable biological material

25. The method of Claim 24, wherein the amount of active contaminants in the membranous freeze dried biological material is reduced to none.

26. The method of anyone of Claims 24 - 25, wherein the membranous dried biological material comprises RBC.

27. The method of anyone of Claims 24 - 26, wherein the membranous dried biological material comprises platelets.

28. The method of anyone of Claims 24 - 26, wherein the membranous dried biological material is essentially free of nucleotide-containing biological material.

29. The method of anyone of Claims 24 - 28, wherein the membranous dried biological material is membranous freeze dried biological material.

30. Biological material obtainable by the method of any one of Claims 24 - 28.

31. The biological material of Claim 30, comprising at least one of RBC or platelets.

32. Dry membranous biological material being essentially free of active contaminants.

33. The dry membranous biological material of Claim 32, comprising RBC.

34. The lyophilized membranous biological material of anyone of Claims 32 or 32, wherein the biological material comprises platelets.

35. The dry membranous biological material of anyone of Claims 32 to 34 comprising a polyphenol.

36. The dry membranous biological material of Claim 35, wherein the polyphenol is EGCG.

37. The dry membranous biological material of anyone of Claims 32 to 36, wherein the dry membranous biological material is lyophilized membranous biological material.

Figure 1

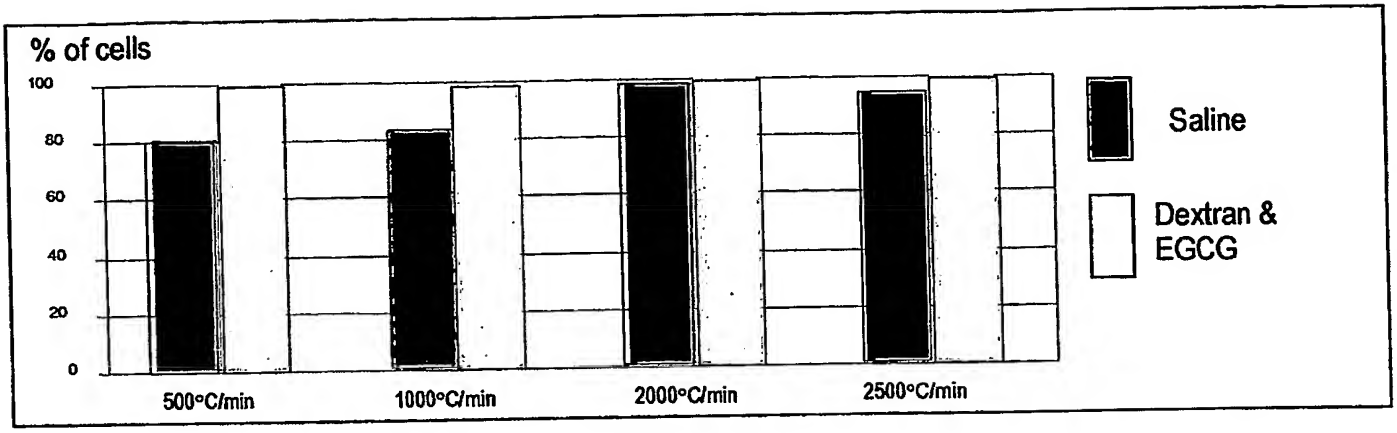


Figure 2

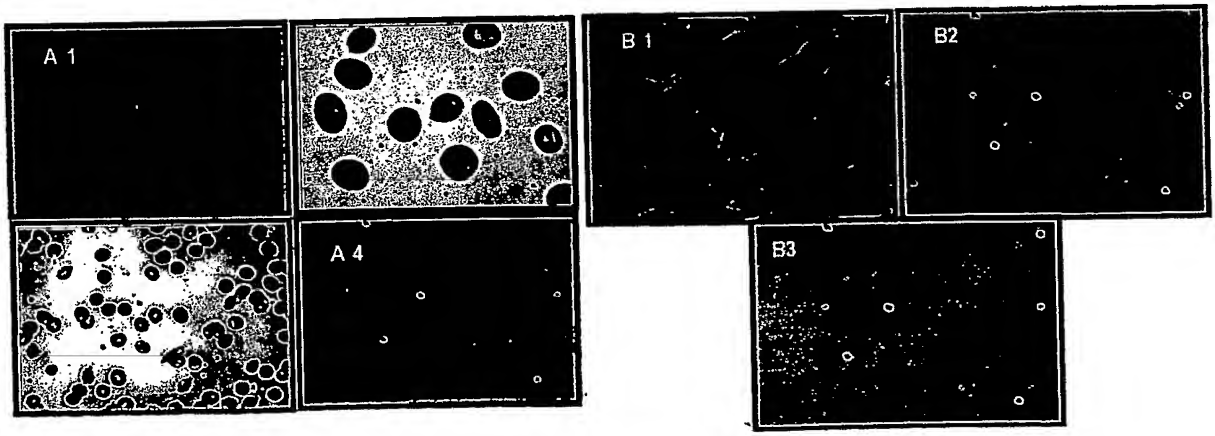


Figure 3

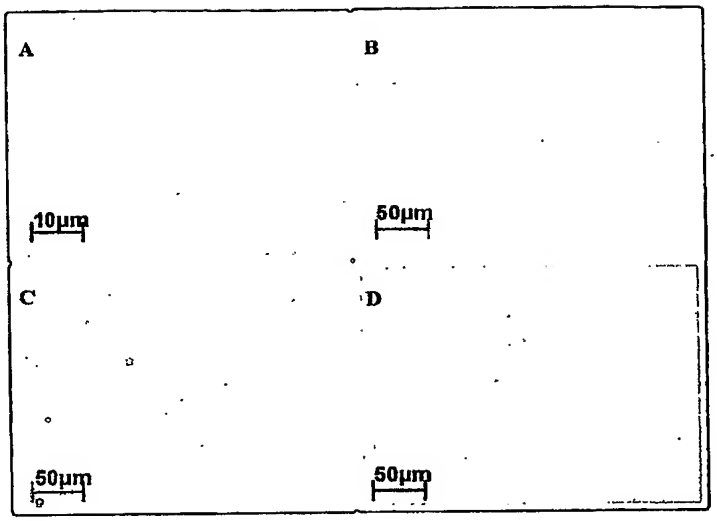


Figure 4

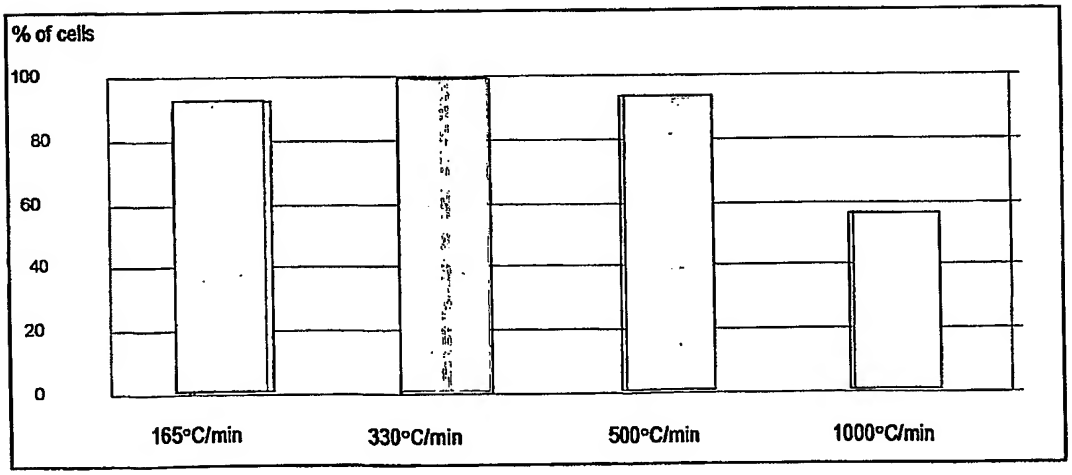


Figure 5

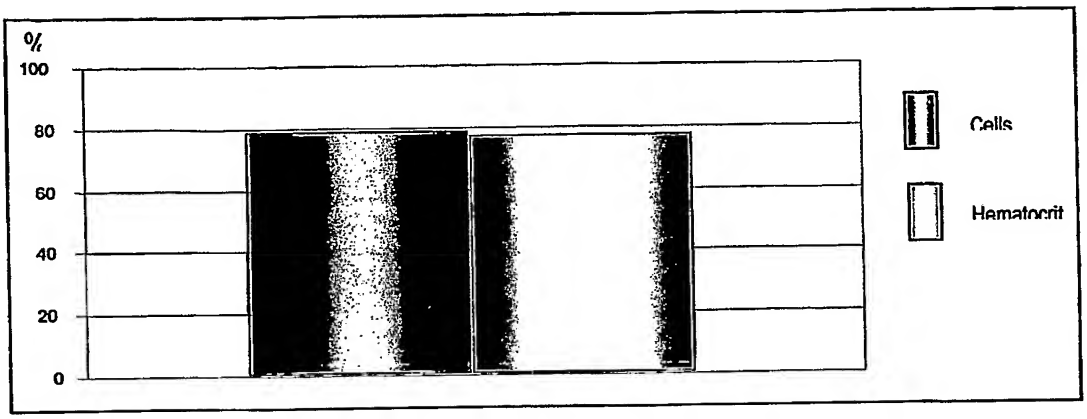


Figure 6

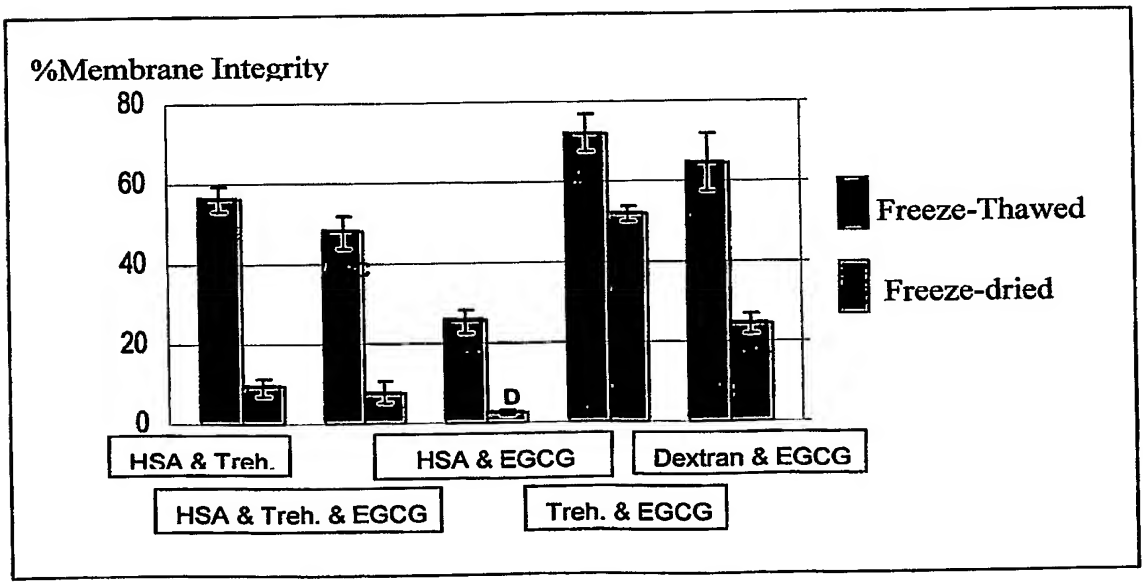
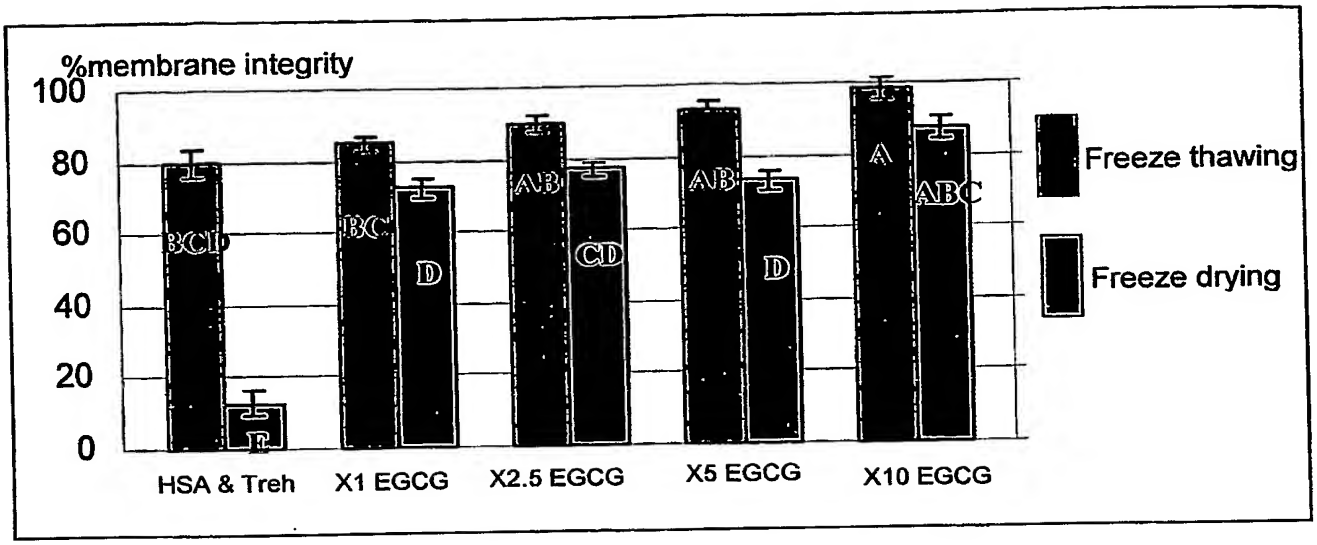


Figure 7



01-25\01524650